Appl Biochem Biotechnol (2011) 164:804–818
DOI 10.1007/s12010-011-9175-5

Phosphoproteome Profiling Using a Fluorescent Phosphosensor Dye in Two-Dimensional Polyacrylamide Gel Electrophoresis

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Received: 18 August 2010 / Accepted: 18 January 2011 /
Published online: 8 March 2011

Abstract We validated the novel PhosphoQUANTI SolidBlue Complex (PQSC) dye for the sensitive fluorescent detection of phosphorylated proteins in polyacrylamide- and two-dimensional gel electrophoresis (PAGE and 2DE, respectively). PQSC can detect as little as 15.6 ng of ß-casein, a pentaphosphorylated protein, and 61.3 ng of ovalbumin, a diphosphorylated protein. Fluorescence intensity correlates with the number of phosphorylated residues on the protein. To demonstrate the specificity of PQSC for phosphoproteins, enzymatically dephosphorylated lysates of Swiss 3T3 cells were separated in 2DE gels and stained by PQSC. The fluorescence signals in these gels were markedly reduced following dephosphorylation. When the phosphorylated proteins in Swiss 3T3 cell lysates were concentrated using a phosphoprotein enrichment column, the majority of phosphoproteins showed fluorescence signals in the pI 4–5 range. Finally, we performed phosphoproteome analysis to study differences in the protein phosphorylation profiles of proliferating and quiescent Swiss 3T3 cells. Over 135 discernible protein spots were detected, from which a selection of 15 spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS). The PQSC staining procedure for phosphoprotein detection is simple, reversible, and fully compatible with MALDI TOF-MS.

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Keywords Phosphoproteome · Two-dimensional PAGE · MALDI TOF-MS · PhosphoQUANTI solidblue complex · Swiss 3T3 cell

Abbreviations

2DE	Two-dimensional gel electrophoresis
MALDI TOF-MS	Matrix-assisted laser desorption ionization mass spectrometry
PQSC	PhosphoQUANTI SolidBlue Complex
CBB	Coomassie brilliant blue
FCS	Fetal calf serum

Introduction

Currently one of the most utilized approaches in proteomics includes two-dimensional gel electrophoresis (2DE) and mass spectrometry to quantify and identify proteins [1]. Recent developments, including improved protein solubilization, enhanced 2DE resolution, and increased capabilities of modern mass spectrometry to sequence peptides, have made proteomics a critical research tool in biological sciences [2–4]. Therefore, 2DE is widely used in the proteome because it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms, or post-translational modifications.

Detection of post-translational modifications requires the labeling process to have much higher specificity and sensitivity than is necessary for total protein labeling. Indeed, many regulatory and signaling proteins are extremely hard to detect using traditional methods. Protein phosphorylation is one of the most important cellular regulatory modifications, which occurs via transmembrane receptors [5, 6] as well as in several intracellular signaling pathways. Thus, global analysis of phosphorylation patterns is of crucial importance in understanding signaling processes, and development of methods for phosphoproteome profiling is a major challenge of proteomics. Existing techniques use phosphosite-specific antibodies [7], isotope metabolic labeling [8], and chemical modifications of phosphorylated residues [9]. The phosphoprotein sensor dye, Pro-Q Diamond, is well regarded for phosphoproteome analysis [10]. The fluorescent dye provides global and direct detection of phosphorylated proteins, in contrast to antibodies and metabolic ([^{32}P] orthophosphate) labeling techniques which provide only indirect detection [11, 12]. Radio-labeling methods have the additional drawback that they are only effective in labeling proteins phosphorylated during the procedure, missing those proteins phosphorylated prior to the labeling step. However, there are a few choices in the phosphoprotein sensor dye for 2DE proteome analysis without Pro-Q Diamond. Hence, we propose here an alternative fluorescent dye that is also capable of sensitive and specific detection of phosphorylated amino acid residues in proteins, in conjunction with separation by gel electrophoresis. Recently, a new type of fluorescent chemosensor, PhosphoQUANTI SolidBlue Complex (PQSC), has been developed which can distinguish phosphorylated from non-phosphorylated peptides [13, 14]. Using this PQSC dye, we have previously reported differences in hippocampal phosphoprotein expression profiles in normal and human mutant tau transgenic mice using 2DE analysis [15].

Here, we used 2D gel electrophoresis to separate crude protein lysates from stimulated Swiss 3T3 cells and detected phosphoproteins using PQSC fluorescent dye. We demonstrate that using PQSC and 2DE is an efficient method of functional proteomic analysis that can be used to study signal transduction pathways in Swiss 3T3 cells.

Materials and Methods

Materials

Chicken egg ovalbumin, β-casein, bovine serum albumin (BSA), and lysozyme for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or 2DE were all from Sigma-Aldrich (St. Louis, MO, USA). Source information for all other assay reagents and materials are incorporated into their respective assay methods described below.

Cell Culture

Swiss 3T3 cells were obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). Cells were maintained in DMEM containing 10% fetal calf serum (FCS). For assay, Swiss 3T3 cells were plated into 100-mm dishes and grown to approximately 90% confluence. Media was then replaced with fresh media containing 0.1% FCS and grown for a further 24 h. After washing three times with PBS, cells were incubated in 10 ml fresh media either with or without serum for a further 30 min prior to lysis and sample preparation.

Sample Preparation

After removal of the culture medium, the cells (on ice) were washed three times with ice-cold PBS (Mg^{2+}-free), scraped into 1 ml PBS, transferred to a 1.5-ml tube, centrifuged (15,000×g, 5 min at 4 °C), resuspended in 100 μl of lysis buffer (7 M urea, 2 M thiourea, 5% CHAPS, 2% IPG buffer (GE Healthcare UK Ltd., England), 50 mM 2-mercaptoethanol, 2.5 μg/ml DNase I, 2.5 μg/ml RNase A), and disrupted by sonication for 30 s. Lysate was again centrifuged (15,000×g, 30 min) to remove cellular debris and the supernatant recovered for use in 2DE.

Phosphoprotein Separation and Detection Procedures

2DE was carried out using the method of Gorg et al. [16] and Toda et al. [17] with modification as described previously [18]. For the first-dimension isoelectric focusing gel, Immobiline DryStrip pH 4–7 (7 cm) (GE Healthcare UK Ltd., England), was immersed, and sample lysate applied to the gel. Approximately 100–250 μg of protein was separated at 50 V for 6 h, at 100 V for 6 h, and at 2,000 V for 6 h. After isoelectric focusing, SDS-PAGE was performed with a 12.5% acrylamide gel at 5 mA/gel for 7 h.

For fluorescent staining with PQSC, the SDS-polyacrylamide gel was fixed in a solution containing 10% acetic acid/50% methanol for 30 min, washed (3×10 min) with de-ionized water, incubated in the undiluted stock solution of PQSC (Toyo Ink Co. Ltd., Tokyo, Japan) for 30 min, and de-stained with two successive washes (30 min per wash) of PQSC washing reagent (Toyo Ink Co. Ltd., Tokyo, Japan) containing (or not) 1% Triton X-100. The stained gels were viewed under a UV transilluminator (ATTO, Japan) with 365-nm excitation and 440-nm emission for PQSC dye detection. If required, digital images were acquired using the FluoroPhoreStar 3000 image capture system (Anatech, Japan). Fluorescent staining of the 2DE with Pro-Q Diamond (Molecular Probes, Eugene, OR, USA) was carried out using the method of Steinberg et al. [10]. Images were acquired on an LAS-4000 multicolor image analyzer (Fuji Film, Tokyo, Japan) with 520-nm excitation and 575-nm emission for Pro-Q Diamond dye detection.

Following image acquisition, gels were stained for total protein with Quick Coomassie brilliant blue (CBB) (Wako Pure Chemicals, Osaka, Japan) for serial dichromatic detection,

permitting comparison of the phosphoprotein and total protein profiles. For the comparison of phosphorylation level and protein level in 2DE gels, the image files were analyzed and quantified using PDQuest software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis

Protein levels are expressed as mean ± standard deviation of the percentage of the spot volume in each particular gel after subtraction of the background values. Intergroup differences were analyzed using the non-parametric Mann–Whitney *U* test. The level of significance was set at $P<0.05$.

Phosphoprotein Enrichment

Phosphoproteins from 3T3 cell lysates were enriched using Pro-Q Diamond Phosphoprotein Enrichment Kit made up to 400 μl in lysis buffer, centrifuged at 14,000×g for 10 min and the supernatant transferred to a new tube. The 500 μl of lysate was further diluted to 5 ml in wash buffer and applied to the phosphoprotein enrichment column. After washing the column (3× 1 ml of wash buffer), trapped phosphoproteins were eluted with elution buffer (5×250 μl). The eluted and flow-through fractions were precipitated by ReadyPrep 2-D cleanup kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the precipitates dissolved and applied to 2DE gels.

Lambda Phosphatase Treatment

Swiss 3T3 cells were plated in three 100-mm culture dishes as for the "Cell culture" procedure described above. When cells had grown to subconfluence, they were washed three times with PBS, and the cells lysed in 1 ml of RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). After sonication for 30 s, the lysates were incubated with 10,000 U of lambda protein phosphatase (New England Biolabs Ltd., UK) in 1× Lambda PPase reaction buffer (New England Biolabs Ltd., UK), with or without 0.1 mM $MnCl_2$ for 2 h. The phosphatase-treated lysates were precipitated by ReadyPrep 2-D cleanup kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in accordance with the manufacturer's instructions, and the precipitates dissolved and applied to 2DE gels.

Sample Preparation for Mass Spectrometry Analysis

After protein detection in polyacrylamide gels stained with PQSC and CBB, protein spots were subjected to trypsin digestion. The in-gel digestion was carried out [19] with 31.25 ng porcine trypsin of Promega (Madison, WI, USA). Briefly, gel pieces were washed, dehydrated, and diluted in 200 μl of ammonium hydrogen carbonate 25 mM with 5% acetonitrile (ACN) (*v*/*v*). Five microliters of trypsin 10 ng/μl was added, and the digestion was incubated during 10 h at 37 °C. After separation of supernatant, gel pieces were washed again and then extracted with 50/50 ACN/trifluoroacetic acid 0.3% (v/v) during 10 min by sonication. The supernatant was once again collected, mixed with the two first, and evaporated under vacuum. The extracted peptides were then diluted in 5 μl of 50/50 ACN/trifluoroacetic acid 0.3% (*v*/*v*).

Mass Spectrometry Analysis and Protein Identification

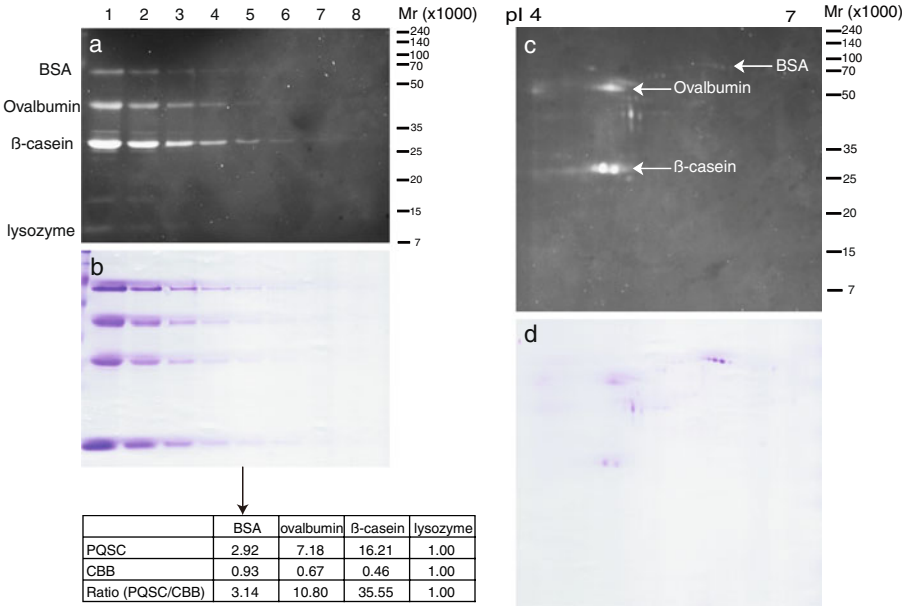
Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was carried out on an Ultraflex time-of-flight (TOF)/TOF mass spectrometer (Bruker Daltonics, Bremen,

Germany) with an accelerating voltage of 25 kV. A peptide solution of 0.7 μl was mixed on target with 0.7 μl of 1 μg/μl α-cyano-4-hydroxycinnamic acid (HCCA; Wako Junyaku, Osaka, Japan) solution in 50/50 ACN/trifluoroacetic acid 0.3% (*v*/*v*). Typically, mass spectrometric spectra were obtained at a laser power of 3,000 kW cm^{-2} and the number of laser shot of 2,500 with HCCA. All MALDI spectra profiles were externally calibrated using peptide calibration standard II (Bruker Daltonics, Germany). Identification of proteins from MALDI/mass spectrometry (MS) spectra was achieved using peptide mass fingerprint of Mascot (http://www.matrixscience.com) website [1] with the ion list from each experiment and [2] with a mixed list containing non-redundant ions. Monoisotopic peptide masses were matched against the Swiss-Prot non-redundant database using 20 or 50 ppm mass tolerance, limited to the Mus Musculus proteins and with a minimum of matched peptides of five. Alkylation of cysteines by acrylamide was considered, but no missed cleavage was allowed.

The identified proteins were searched for putative phosphorylation sites by Scansite 2.0 (http://scansite.mit.edu/). The identified proteins were searched for putative phosphorylation sites by PhosphoSitePlus (http://www.phosphosite.org/homeAction.do).

Western Blot

A protein load of 50 μg/well underwent electrophoresis on a 12.5% sodium SDS-PAGE mini gel subsequent to dilution in 1× Laemli buffer with prior denaturation at 95 °C for



	BSA	ovalbumin	ß-casein	lysozyme
PQSC	2.92	7.18	16.21	1.00
CBB	0.93	0.67	0.46	1.00
Ratio (PQSC/CBB)	3.14	10.80	35.55	1.00

Fig. 1 The sensitivity of PQSC detection of phosphoproteins in SDS-polyacrylamide gels and 2DE gels. Serial dilutions of the phosphoproteins (β-casein and ovalbumin) and non-phosphoproteins (lysozyme and BSA) were separated by electrophoresis and stained with **a** PQSC dye for phosphorylation or **b** CBB for serial total protein detection. **a**, **b** Lanes *1*–*8*: 2,000; 1,000; 500; 250; 125; 62.5; 31.3; and 15.6 ng per lane, respectively. Table shows the ratio of signal intensity in lane *2* (1,000 ng) of SDS-PAGE gels stained by PQSC or CBB. **c**, **d** BSA, ovalbumin and β-casein (1,250 ng) were separated by 2DE gel electrophoresis. Image acquisition was performed on Anatech image capture system FluoroPhoreStar 3000 with exposure time set for 4 s for PQSC. Experiments were carried out five times. Images presented here are representative of these five replicates

10 min. The protein was then transferred to a FluoroTrans™ membrane (0.2 μm) (Pall Corporation, FL, USA) followed by blocking of the membranes with 5% skim milk–PBS for 1 h at room temperature and probed overnight at 4 °C by incubation with the primary antibody, either anti-14-3-3 zeta (phospho S58) rabbit antibody, anti-HSP90 beta (phospho S254) rabbit antibody (Abcam plc, Cambridge, UK), anti-phospho-Vimentin (ser82) rabbit antibody (Cell Signaling Technology, MA, USA), or anti-β-actin mouse antibody (Santa Cruz Biotechnology, CA, USA). The blot was washed and then incubated with an HRP conjugated anti-rabbit whole IgG as the secondary antibody. Bands were developed by chemiluminescent detection reagent ECL Plus (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions.

Results and Discussion

PQSC Discriminates Between Phosphorylated and Non-Phosphorylated Proteins in SDS-PAGE and 2DE Gels

To demonstrate the selectivity and sensitivity of the PQSC phosphoprotein stain, we used a protein mixture containing β-casein (phosphorylated on five residues per protein), ovalbumin (phosphorylated on two residues), and BSA and lysozyme (both non-phosphorylated). This mix underwent a series of twofold dilutions to give 2,000; 1,000;

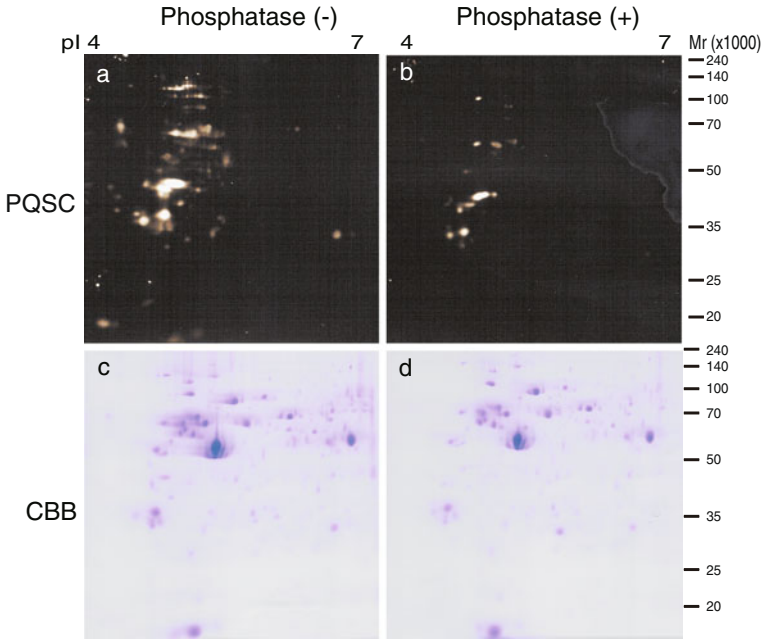


Fig. 2 Monitoring changes in the phosphorylation state of Swiss 3T3 proteins following dephosphorylation with lambda protein phosphatase. **a**, **b** Monitoring the dephosphorylation of Swiss 3T3 proteins incubated with or without lambda phosphatase. Phosphoproteins are stained with PQSC dye. **c**, **d** Visualization of the total proteins profile in a parallel gel using CBB protein gel stain. Image acquisition was performed on a UV transilluminator with a 365-nm filter and an ATTO image capture system (4-s exposure). Experiments were performed three times independently. Representative gel images are shown

500; 250; 125; 62.5; 31.3; and 15.6 ng of each protein per band. These diluted samples were then loaded onto the gel, subjected to electrophoresis, stained with PQSC phosphosensor dye, and imaged (Fig. 1a). The gel was then re-stained with CBB gel stain and imaged a second time (Fig. 1b). On the basis of comparison of Fig. 1a, b, PQSC stain was determined to bind preferentially to the phosphorylated proteins (β-casein and ovalbumin) as opposed to the non-phosphorylated proteins (BSA and lysozyme). A quantitative comparison of total protein and phosphoprotein staining was made using an equal amount of the four-protein mixture stained with PQSC (Fig. 1a) and then by CBB (Fig. 1b). Gel profiles were obtained with ImageJ software (NIH). The band intensity of the BSA, ovalbumin, β-casein, and lysozyme bands (1,000 ng of each) after the phosphoprotein stain was determined to be 2.92, 7.18, 16.21, and 1.00, respectively (normalized to lysozyme signal). The signal intensity of the bands following total protein stain was determined to be 0.93, 0.67, 0.46, and 1.00, respectively (normalized to lysozyme signal). Thus, the phosphoprotein selectivity vs non-phosphorylated protein (i.e., the ratio of the β-casein signal and BSA or lysozyme signals) was 11.32 for BSA and 35.55 for lysozyme. This selectivity of PQSC was sufficient to discriminate non-phosphorylated and phosphorylated proteins.

Fluorescent signals were observable for β-casein at a protein level of as little as 15.6 ng (Fig. 1a, lane 8). For ovalbumin, this figure was 61.3 ng (Fig. 1a, lane 6). By contrast, the non-phosphorylated proteins lysozyme and BSA were not (or only faintly) stained with PQSC, even at high protein concentrations. Ovalbumin and casein have two and five phosphorylation sites, [20, 21] respectively while BSA and lysozyme have none. There is thus a clear correlation between the amount of protein, the number of phosphorylation sites, and the resultant fluorescent signal. Figure 1b, d shows that CBB does not discriminate

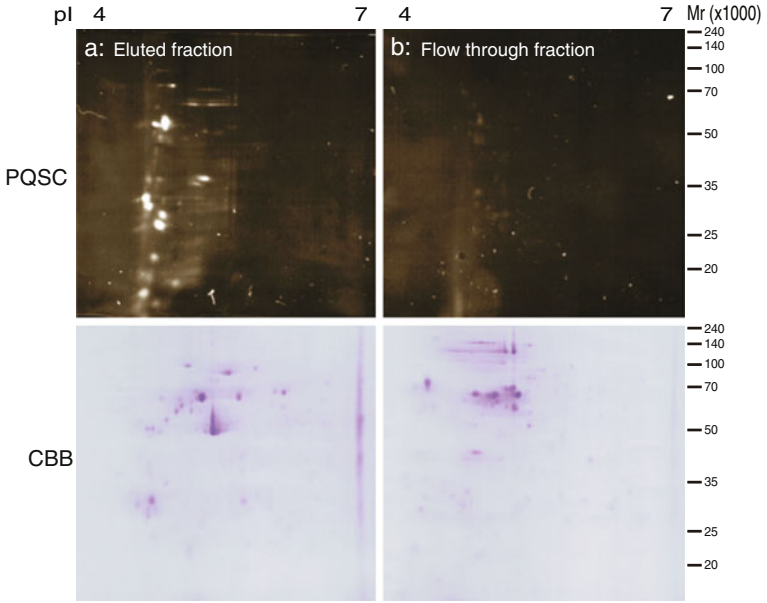


Fig. 3 Specificity of phosphoprotein detection in 2DE gels using PQSC. **a** Eluted fractions of Swiss 3T3 phosphoprotein (250 μg) from Pro-Q Diamond Phosphoprotein Enrichment Kit (Invitrogen, CA, USA) visualized in 2DE gels using PQSC and CBB gel stain. **b** Flow-through fractions from Pro-Q Diamond Phosphoprotein Enrichment Kit visualized in 2DE gels using PQSC and CBB gel stain. Experiments were performed three times independently, and representative of gel images are shown

between phosphorylated and non-phosphorylated proteins, staining both equally. These results demonstrate that PQSC distinguishes phosphorylated from non-phosphorylated proteins in SDS-polyacrylamide gels, and that the intensity of subsequent staining by CBB provides a measure of total protein expression level.

Monitoring Phosphorylation Changes in Swiss 3T3 Proteins Using Lambda Protein Phosphatase

To eliminate the possibility of false positive phosphoprotein staining by PQSC, we enzymatically removed phosphate groups from proteins in the 3T3 lysate and tested the resultant "phosphate-stripped" lysate using 2DE with PQSC dye staining. Lambda protein phosphatase [22] treatment decreased PQSC dye staining of Swiss 3T3 proteins relative to controls (Fig. 2a, b). Subsequent staining with CBB (Fig. 2c, d) demonstrated that the loss of PQSC signal shown in Fig. 2b was not due to proteolysis of the phosphoproteins. The loss of fluorescent signal following lambda phosphatase treatment further supports a phosphorylation-specific activity of the PQSC dye. Although the phosphatase treatment could cause a limited degree of reduction in the PQSC dye binding, this could be explained by the lamda phosphatase which may incompletely degrade the phosphates of proteins.

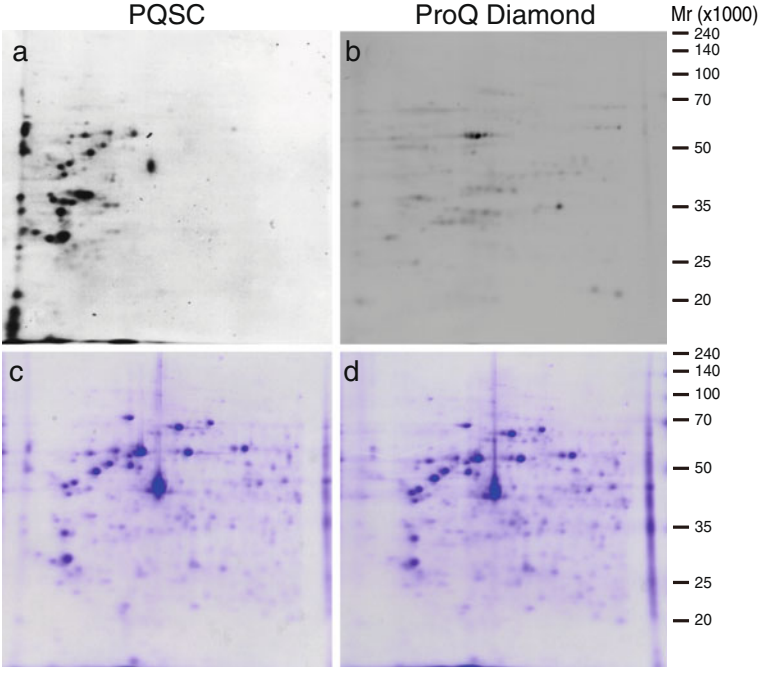


Fig. 4 Two-dimensional gel detection of phosphoproteins from a complex cell lysate using PQSC compared with Pro-Q Diamond. **a** 2D gel electrophoresis of a lysate of Swiss 3T3 cells (150 μg of protein) stained with PQSC and imaged using a UV transilluminator (365 nm) and an ATTO image capture system with exposure time set for 4 s. The PQSC-stained gel image was changed by reversal. **b** 2D gel electrophoresis of a lysate of Swiss 3T3 cells (150 μg of protein) stained with Pro-Q Diamond and imaged using a blue light emitting diode set at 520 nm (excitation) with a 575-nm-long pass filter (emission). Image acquisition was performed by a Fuji LAS-4000 multicolor image analyzer with exposure set for 3 min. **c**, **d** Same gels after CBB staining; total proteins detected

Phosphoprotein Enrichment Profiling in Swiss 3T3 Proteins by PQSC

To profile the phosphoproteome of Swiss 3T3 cells, phosphoprotein enrichment and 2DE were performed on Swiss 3T3 cell lysates. Cell lysate was passed over phosphoprotein affinity

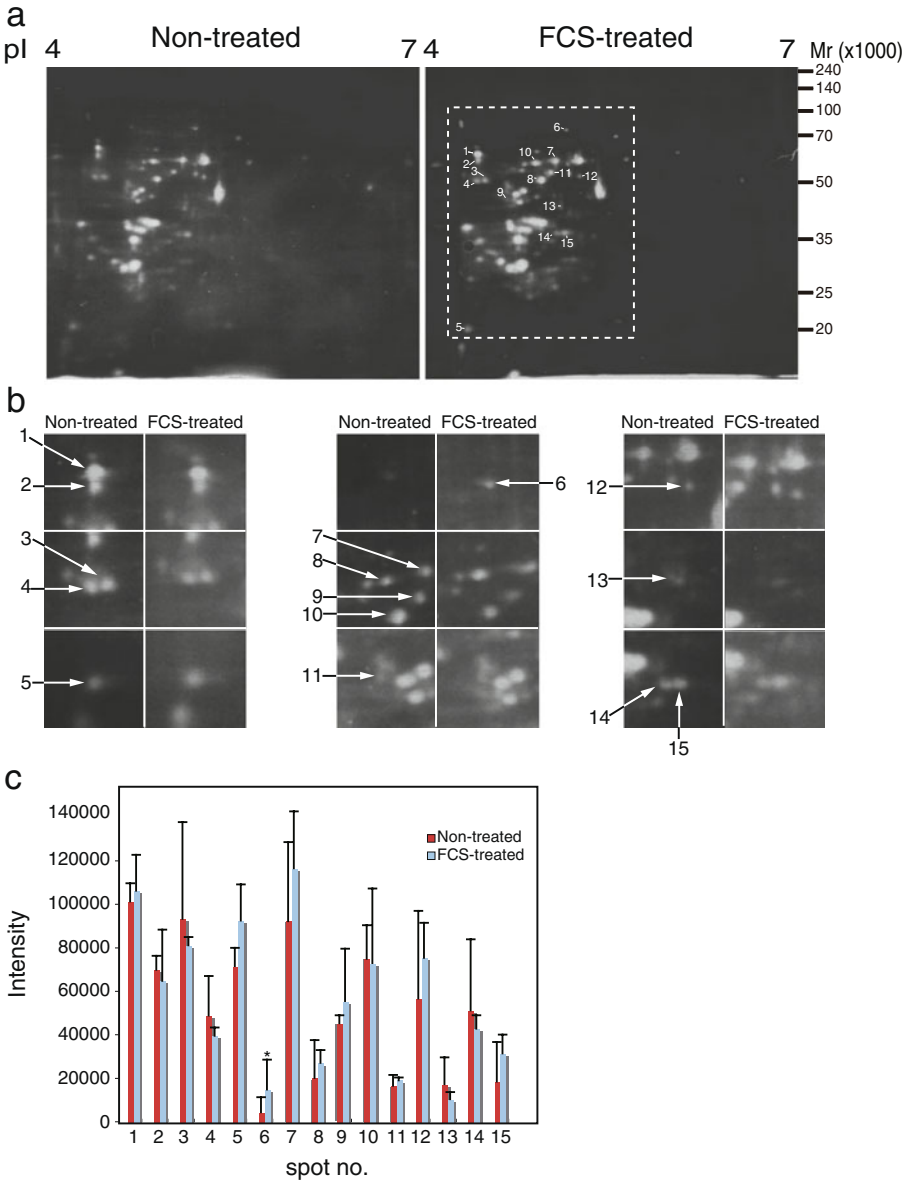


Fig. 5 Two-dimensional gel electrophoretic analysis of FCS-treated Swiss 3T3 cells. **a** Images of representative (n=3) PQSC-stained 2DE gels obtained by fractionating 200 μg of lysate. **b** Magnified regions of the 2DE gels of expressed spots among non-treated and FCS-treated Swiss 3T3 cells. *Arrowheads* indicate the identified spots. **c** The densities were quantified (n=3; mean ± SD) as described in "Materials and Methods," and plots of these densities were compared for the two lysates (serum-starved vs. FCS-treated cells)

columns, and eluted proteins were then detected by PQSC and CBB staining. Most spots were present at low isoelectric points in the pI 4–5 range by PQSC staining (Fig. 3). Similar results were obtained previously showing the majority of Pro-Q staining of phosphoproteins in a monocyte phosphoproteome to be enriched in the pI 4–5.5 range [23]. In the eluted fraction (Fig. 3a, upper and lower panels), PQSC mainly stained spots in the pI 4–5 range that gave weak signals under CBB staining. There was very little PQSC staining in the pI 6–7 range (Fig. 3b, upper), despite significant staining by CBB in this region (Fig. 3a, lower). In the flow-through fraction, almost all proteins ran in the pI 4–6 range (Fig. 3b, lower) but very few of these proteins were stained with PQSC. These results suggest that Swiss 3T3 protein lysates were successfully concentrated by the Pro-Q Diamond Phosphoprotein Enrichment Kit, but that those proteins that reacted with PQSC were almost all in the pI 4–5 range, with any trapped phosphoproteins in the pI 6–7 range reacted only weakly with PQSC.

Comparison of PQSC and Pro-Q Diamond Phosphoprotein Staining in 2DE Gels

To determine whether PQSC could be used to detect the phosphorylated proteins in 2DE, we compared the ability of PQSC and Pro-Q Diamond to stain phosphoproteins in 2DE. Figure 4 compares the 2DE profiles of phosphoproteins from Swiss 3T3 cell lysates stained with PQSC and Pro-Q Diamond. There were 135 and 118 phosphoprotein spots revealed by

Table 1 Identification of 15 proteins from Swiss 3T3 cell lysates by MALDI TOF-MS

Spot no.	Description	Swiss-Prot ID	Score[a]	Sequence coverage (%)	MW practical (theoretical)	pI practical (theoretical)
1	Calumenin	CALU_MOUSE	232	16	60,000 (37,041)	4.2 (4.49)
2	HSP 90 B	HS90B_MOUSE	221	6	55,000 (83,621)	4.2 (4.97)
3	Endoplasmin	ENPL_MOUSE	233	10	48,000 (92,708)	4.2 (4.74)
4	Vimentin	VIME_MOUSE	422	25	47,000 (53,713)	4.1 (5.06)
5	Fbxo2 protein	gi 20,072,543	58	13	23,000 (33,042)	4.1 (4.27)
6	Nascent polypeptide-associated complex subunit	NACA_MOUSE	99	19	75,000 (23,370)	5.2 (4.52)
7	Tropomyosin alpha-3 chain	TPM3_MOUSE	239	15	55,000 (32,901)	5.0 (4.68)
8	Tropomyosin alpha-1 chain	TPM1_MOUSE	96	7	48,000 (32,719)	4.8 (4.69)
9	HSC70-interacting protein	F10A1_MOUSE	82	9	42,000 (41,804)	4.5 (5.19)
10	Complement component 1 Q subcomponent-binding protein	C1QBP_MOUSE	120	9	53,000 (31,342)	4.6 (4.82)
11	Elongation factor 1-beta	EF1B_MOUSE	115	6	54,000 (24,852)	4.5 (4.53)
12	14-3-3 Protein zeta	1433Z_MOUSE	325	31	50,000 (27,928)	5.4 (4.73)
13	Ras-related protein Rap-1A	RAP1A_MOUSE	59	6	40,000 (21,322)	5.1 (6.38)
14	Calmodulin-alpha	CALMA_ARBPU	134	11	38,000 (16,037)	5.0 (4.04)
15	Nucleophosmin	NPM_MOUSE	93	11	36,000 (32,714)	5.2 (4.62)

Swiss 3T3 cells proteins were separated by 2DE and identified by MALDI TOF-MS, following in-gel digestion with trypsin. The spots representing the identified proteins are indicated in Fig. 4 and are designated with the accession IDs assigned to them in the Swiss-Prot database

[a] Score relates to the probability assignment for each protein. Score and sequence coverage were calculated using the MASCOT search engine (http://www.matrixscience.com)

PQSC and Pro-Q Diamond. However, the phosphoprotein staining profiles for PQSC and Pro-Q Diamond in 2DE gels were quite different. Optimal separation (the maximum number of spots) was apparent by Pro-Q Diamond staining at pI 4–7 and by PQSC staining at pI 4–5.5. PQSC is a bis(zinc(II)-dipicolylamine)-based artificial receptor that strongly binds to negatively charged phosphorylated peptides. It distinguishes phosphorylated from non-phosphorylated peptides, and the fluorescence intensity depends on the net charge of the phosphorylated peptides [13, 14], suggesting that the ability of PQSC staining to distinguish between individual phosphoproteins in 2DE gels depends on the net negative charge of the phosphoproteins.

Pro-Q Diamond could also specifically stain to phosphoproteins in 2DE [24], the structure based on the Ca^{2+} indicators fluorescein chromophores in which the chelating site directly includes the xanthene chromophere [13]. The difference between the staining abilities of PQSC and Pro-Q Diamond is unclear but may have a structural basis.

PQSC Quantified the Change in 2DE from FCS-Treated Swiss 3T3 Cell Lysate

A critical downstream application for any new staining method is the facility to perform subsequent analysis by MS for identification of the protein under investigation. We analyzed the changes in phosphoprotein expression in FCS-treated Swiss 3T3 cells using the PQSC phosphosensor dye. Cell lysates prepared from untreated and 10% FCS-treated cells were fractionated on 2DE gels to visualize differences in their respective proteomes.

Table 2 Identification of putative phosphorylation residues by Scansite

Spot no.	UniProtKB ID (Swiss-Prot ID)	Phosphorylation residues identified by ScanSite (certified by references (or similarity) in UniProtKB)
1	O35887 (CALU_MOUSE)	T^{65} (similarity)
2	P11499 (HS90B_MOUSE)	S^{226}, S^{255}, S^{261}, T^{297}, Y^{305}, S^{307}, S^{452}, Y^{484}, S^{532}, S^{718}
3	P08113 (ENPL_MOUSE)	S^{306}
4	P20152 (VIME_MOUSE)	S^{7}, S^{9}, S^{10}, S^{21}, S^{25}, S^{26}, S^{34}, S^{39}, S^{42}, S^{47}, S^{51}, Y^{53}, Y^{61}, S^{66}, S^{72}, S^{73}, S^{82}
5	ND (gi 20072543)[a]	ND[b]
6	Q60817 (NACA_MOUSE)	S^{43}, T^{159}, T^{161}, S^{166}
7	P21107 (TPM3_MOUSE)	S^{283}
8	P58771 (TPM1_MOUSE)	S^{283}
9	Q99L47 (F10A1_MOUSE)	S^{74}, S^{75}, S^{78}, S^{180} (similarity)
10	O35658 (C1QBP_MOUSE)	Y^{184}, S^{197} (similarity)
11	O70251 (EF1B_MOUSE)	S^{83}, S^{106}
12	P63101 (1433Z_MOUSE)	S^{58}, S^{184}, S^{232} (similarity)
13	P62835 (RAP1A_MOUSE)	ND[b]
14	P62146 (CALMA_ARBPU)	ND[b]
15	Q61937 (NPM_MOUSE)	S^{252}, S^{258}

The identified proteins were searched for putative phosphorylation sites by Scansite (http://scansite.mit.edu/). The residues were certified by references or, if direct evidence was lacking, by similarity to related proteins (as indicated)

[a] ND denotes that the Swiss-Prot ID number was not recognized in the Scansite database

[b] ND denotes that no phosphorylation residues could be certified either by references or similarity in UniProtKB

Images of PQSC-stained gels were captured using the fluorescence image capture system (ATTO, Japan) and differentially phosphorylated protein spots were identified using PDQuest as described in the "Materials and Methods" section. Of 135 discernible protein spots (Fig. 5a), 17 protein spots were randomly selected and identified by MALDI TOF-MS, protein identification was performed by searching extracted peak lists against the NCBInr database using the Mascot search engine. As a result, 15 proteins were identified and described in Table 1 except two "unnamed proteins" showing Mascot scores under 50. One spot (spot 6; Fig. 5b) showed significantly increased phosphorylation compared to the corresponding spot on the 2DE gel of the non-treated cell lysate, indicating that the protein was differentially phosphorylated in FCS-treated Swiss 3T3 cells (Fig. 5a). For the other 14 spots, there was no difference in spot intensity on 2DE gels between lysates from FCS-treated and non-treated Swiss 3T3 cells (Fig. 5b, c).

Optimal separation (the maximum number of spots) was apparent by PQSC staining at pI 4–5 (Fig. 5a, b). This evidence suggested that PQSC had considerable sensitivity for phosphoproteins in the pI 4–5 range but not those over pI 6 (Fig. 5). It clearly distinguishes phosphorylated from non-phosphorylated peptides, and the fluorescence intensity depends on the net charge of the phosphorylated peptides [13]. Recently, Kinoshita et al. reported a dinuclear metal complex, Phos-tag, which specifically binds phosphoproteins [25]. These reagents have certain structural dissimilarities which might explain the differential staining

Table 3 Identification of putative phosphorylation residues by PhosphoSitePlus

Spot no.	UniProtKB ID (Swiss-Prot ID)	Phosphorylation residues identified by PhosphoSitePlus (certified by references in UniProtKB)
1	O35887 (CALU_MOUSE)	S^{69}
2	P11499 (HS90B_MOUSE)	Y^{56}, Y^{192}, S^{226}, S^{255}, S^{261}, Y^{276}, Y^{484}, Y^{485}, Y^{596}, Y^{619}
3	P08113 (ENPL_MOUSE)	S^{306}, Y^{677}
4	P20152 (VIME_MOUSE)	S^{5},S^{7}, S^{9}, S^{10}, S^{21}, S^{25}, S^{26}, S^{34}, T^{37}, Y^{38}, S^{39}, S^{42}, S^{47}, S^{51}, Y^{53}, S^{55}, S^{56}, Y^{61}, S^{65}, S^{66}, S^{72}, S^{73}, S^{83}, S^{87}, Y^{117}, S^{144}, Y^{150}, S^{205}, S^{226}, Y^{276}, K^{292}, S^{325}, S^{412}, S^{419}, S^{420}, T^{426}, S^{430}, S^{438}, T^{458}, S^{459}
5	ND (gi 20072543)[a]	S^{9}, S^{11}, S^{17}
6	Q60817 (NACA_MOUSE)	ND[b]
7	P21107 (TPM3_MOUSE)	Y^{162}, S^{283}
8	P58771 (TPM1_MOUSE)	S^{45}, S^{87}, Y^{162}, S^{174}, S^{206}, S^{252}, Y^{261}, T^{282}, S^{283}
9	Q99L47 (F10A1_MOUSE)	S^{74}, S^{75}, S^{78}
10	O35658 (C1QBP_MOUSE)	S^{147}, S^{197}
11	O70251 (EF1B_MOUSE)	Y^{79}, S^{82}, S^{83}, T^{87}, T^{88}, S^{90}, S^{106}, S^{112}
12	P63101 (1433Z_MOUSE)	S^{58}, Y^{179}, S^{207}, T^{229}, S^{230}, T^{232}
13	P62835 (RAP1A_MOUSE)	Y^{159}
14	P62146 (CALMA_ARBPU)	ND[b]
15	Q61937 (NPM_MOUSE)	S^{4}, S^{10}, Y^{29}, S^{43}, Y^{67}, S^{70}, T^{75}, S^{82}, S^{106}, S^{112}, S^{125}, S^{139}, S^{194}, T^{198}, S^{216}, T^{217}, T^{232}, T^{235}, S^{241}, S^{252}, S^{258}

The identified proteins were searched for putative phosphorylation sites by PhosphoSitePlus (http://www.phosphosite.org/homeAction.do). These residues were certified by references

[a] ND denotes that the Swiss-Prot ID number was not recognized in the PhosphoSite database

[b] ND denotes that no phosphorylation residues could be certified either by references or similarity in UniProtKB

profiles in 2DE. Phosphoproteome research needs the wide variety of phosphosensor dyes because higher sensitivity and specificity facilitate new discoveries in cellular signal transduction.

Protein Identification by Mass Spectrometry

The single spot showing increased phosphorylation in Swiss 3T3 cells following FCS treatment may be a key protein in signaling or cellular proliferation, so we sought to identify it (and the other 14 selected spots in Fig. 4b) using mass spectrometry. Proteins in these spots were digested with trypsin, and the peptides were analyzed and identified by MALDI TOF-MS (Table 1).

We determined whether the identified proteins contained phosphorylation residues using a Scansite search. These results are described in Table 2. HSP90B, endoplasmin, Vimentin, nascent polypeptide, tropomyosin, and nucleophosmin contain phosphorylation residues validated by the published references in the database of UniProtKB. Calumenin, complement component 1 Q, HSC70-interacting and 14-3-3 protein zeta contain phosphorylation motifs with a significant similarity to related proteins in the UniProtKB database (Table 2). Calmodulin-α, Fbxo2 protein and Rap1A contained no validated phosphorylation sites, nor any similarity to phosphorylation motifs in other proteins.

In addition, these proteins were analyzed using the PhosphoSitePlus database (Table 3). In this analysis, nascent polypeptide-associated complex subunit and calmodulin-α were identified as not having any phosphorylation sites. Though calmodulin-α was not certified

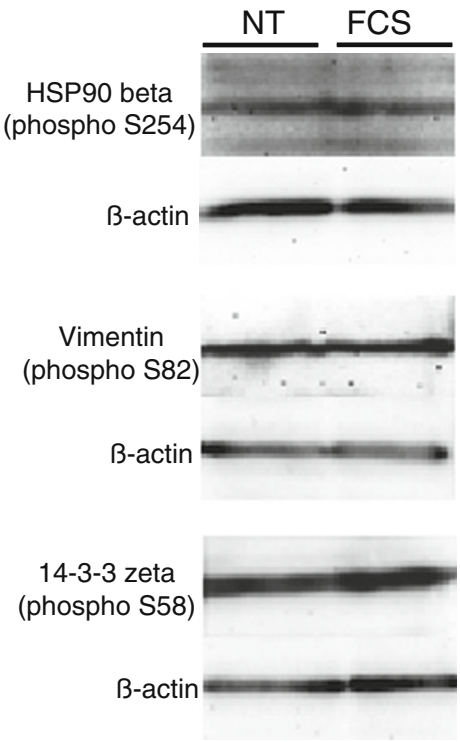


Fig. 6 Validation of phospho-14-3-3 zeta, phospho-HSP90 beta, and phospho-Vimentin by Western blot in non-treated and FCS-treated Swiss 3T3 cells. Total protein (25 μg/well) from Swiss 3T3 lysate underwent electrophoresis on a 12.5% SDS-PAGE and analyzed by Western blotting. Phospho-14-3-3 zeta, phospho-HSP90 beta, and phospho-Vimentin were analyzed in three out of 15 non-treated and FCS-treated Swiss 3T3 cells by 2DE using PQSC. Bands correspond to 28 kDa phospho-14-3-3 zeta, 83 kDa phospho-HSP90 beta proteins, and 57 kDa phospho-Vimentin. β-Actin was used as a loading control in Western blot analysis. *NT* serum-starved cells, *FCS* FCS-treated cells for 30 min

by either database, the majority of the PQSC-reactive proteins were classed as phosphoproteins by at least one of the databases.

Validation of PQSC-Reactive Phosphoproteins by Western Blot

To validate phosphoprotein attained by PQSC, Western blot was performed for 14-3-3 zeta, HSP90 beta, and Vimentin in three out of 13 proteins used in the identified phosphoproteins. The results obtained in the Western blot bands followed a similar regulation to 2DE analysis by using PQSC (Fig. 6); there were no differences of the phosphoprotein level between non- and FCS-treated Swiss 3T3 cells. The 14-3-3 zeta, HSP90 beta, and Vimentin were validated by Western blot analysis using phosphoprotein-specific antibodies. These results reinforced that PQSC could be used as the phosphoprotein sensor dye for 2DE phospho proteome.

Conclusions

In summary, we describe the first use of PQSC as a phosphorylation sensor in a complex mixture of proteins from Swiss 3T3 cells and establish its usefulness for identifying phosphoproteins in 2D gels. PQSC staining in gels provides a direct approach to visualizing the phosphoproteome and is fully compatible with other proteome analysis procedures, such as MALDI TOF-MS. Phosphoproteome analysis is considered to be key strategy to gaining a new understanding of the regulation of biological processes.

Acknowledgments The authors thank Dr. Tymothy Cutler (University of California, San Francisco) for encouraging and proofreading the original manuscript. This work was supported by a grant from Kobe Gakuin University for Collaborative Research B.

Competing Interests None declared.

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